

***Geobacter*, *Anaeromyxobacter* and *Anaerolineae* populations are enriched on anodes of root exudate-driven microbial fuel cells in rice field soil**

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Summary

Plant-based sediment microbial fuel cells (PMFCs) couple the oxidation of root exudates in living rice plants to current production. We analysed the composition of the microbial community on anodes from PMFC with natural rice field soil as substratum for rice by analysing 16S rRNA as an indicator of microbial activity and diversity. Terminal restriction fragment length polymorphism (TRFLP) analysis indicated that the active bacterial community on anodes from PMFCs differed strongly compared with controls. Moreover, clones related to *Deltaproteobacteria* and *Chloroflexi* were highly abundant (49% and 21%, respectively) on PMFCs anodes. *Geobacter* (19%), *Anaeromyxobacter* (15%) and *Anaerolineae* (17%) populations were predominant on anodes with natural rice field soil and differed strongly from those previously detected with potting soil. In open circuit (OC) control PMFCs, not allowing electron transfer, *Deltaproteobacteria* (33%), *Betaproteobacteria* (20%), *Chloroflexi* (12%), *Alphaproteobacteria* (10%) and *Firmicutes* (10%) were detected. The presence of an electron accepting anode also had a strong influence on methanogenic archaea. Hydrogenotrophic methanogens were more active on PMFC (21%) than on OC controls (10%), whereas acetoclastic *Methano-*

***saetaceae* were more active on OC controls (31%) compared with PMFCs (9%). In conclusion, electron accepting anodes and rice root exudates selected for distinct potential anode-reducing microbial populations in rice soil inoculated PMFC.**

Introduction

Soils and sediments containing organic matter have been employed to produce electrical current in microbial fuel cells (MFC) (Reimers *et al.*, 2001; Tender *et al.*, 2002; Donovan *et al.*, 2011). This is possible due to the development of an anodic biofilm that includes microorganisms capable of converting chemical energy directly into electric current (Logan *et al.*, 2006; Lovley, 2006; Davis and Higson, 2007). An increase in current production has been observed when plants are included in the system, probably due to the increase of available organic matter due to the release of root exudates by the plant (Kaku *et al.*, 2008; Strik *et al.*, 2008; De Schamphelaire *et al.*, 2010). Anode microbial communities have been shown to be highly diverse, and several phylogenetic groups have been found to be predominant, determined by the inoculum used (Holmes *et al.*, 2004a), the substrate used for feeding (Jung and Regan, 2007; Chae *et al.*, 2009; Chung and Okabe, 2009; Sun *et al.*, 2010) and the anode material (Liu *et al.*, 2007), among other factors. Only a few studies focused on the anode microbial communities from plant-based sediment microbial fuel cells (PMFC). In a rice-based PMFC with potting soil as substratum, a high abundance of populations related to *Desulfobulbus* spp. and *Geobacter* spp. was detected on anodes (De Schamphelaire *et al.*, 2010). Both genera have been described to produce current in MFCs (Bond *et al.*, 2002; Bond and Lovley, 2003; Holmes *et al.*, 2004b), and therefore might be responsible for current production in PMFCs. In a rice PMFC operated during a field trial in rice paddy, the bacterial community on anodes was analysed by community fingerprinting, but the composition of the bacterial community on anodes was not comprehensively determined (Kaku *et al.*, 2008). More recently, Timmers and colleagues (2012) studied the microbial community that developed in a *Glyceria maxima* PMFC with graphite granules as substratum. The authors suggest that

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Geobacter species are responsible for current generation but found other bacterial groups, such as anaerobic cellulolytic bacteria probably providing substrates for the electroactive bacteria, or denitrifiers probably competing for short-chain fatty acids.

Much less is known about the involvement of archaea in PMFCs. In rice PMFCs with potting soil, not only methanogens were detected but a group of uncultured Euryarchaea was prominent on the anode (De Schamphelaire *et al.*, 2010). In a rice PMFC with graphite granules and vermiculite, *Methanomicrobiales* and *Methanobacteriales* were found dominant, suggesting H₂ was a more important substrate for methanogenesis than acetate (Arends *et al.*, 2014).

In the present study, we identified the active bacterial and archaeal communities based on the analysis of 16S rRNA on rice PMFC with rice field soil as substratum for the rice plant. We hypothesize that using rice field soil – the natural substratum for rice plants – rather than potting soil (De Schamphelaire *et al.*, 2010) or artificial substrata (e.g. vermiculite, graphite granules (De Schamphelaire *et al.*, 2010; Arends *et al.*, 2014) has a strong effect on the active anode microbial communities as the underlying microbial seed bank can have a profound effect on the development of the microbial community composition in the rhizosphere (Conrad *et al.*, 2008). We used rRNA as a measure of diversity of the metabolically active members of a community, as rRNA is more labile and ribosome numbers have been correlated with cellular activity (Kramer and Singleton, 1993; Lee and Kemp, 1994), while DNA-based analysis captures the presence of populations only, including dormant populations or even dead cells (Mengoni *et al.*, 2005). The identification of the microorganisms involved in bioelectrochemical systems is an essential step for understanding the factors that govern PMFC performance.

Results and discussion

Electrochemical performance

In order to study the active anode microbial populations developed in rice PMFC with rice soil, we constructed and operated two series (A and B) of duplicate rice soil PMFC for 104 and 90 days in 2007 and 2008 respectively. Two open circuit (OC) controls (one for each series) and one unplanted (no plant, NP) control for series A were constructed and operated under the same conditions as the PMFCs (for detailed description, see Appendix S1). The electrical potential of the PMFCs was measured along the operation time (Fig. S1), and the current production of the PMFCs was twice the current production of the unplanted control during the stable period [days 40–100, PMFC-A, 15 ± 1 mA m⁻² total

anode surface (TAS); PMFC-B, 18 ± 1 mA m⁻² TAS; NP-A, 8 ± 1 mA m⁻² TAS], and OC potentials attained up to 900 mV. The current output values were in the range of average current densities reported in literature for PMFCs (Kaku *et al.*, 2008; Strik *et al.*, 2008; De Schamphelaire *et al.*, 2010; Timmers *et al.*, 2012). The potential increase at day 41 and the difference observed between PMFC and the unplanted control indicated that most likely root exudates released by the plant increased current production, and thereby improved the performance of the PMFCs.

Active bacterial and archaeal populations on PMFC anodes

The composition of bacterial and archaeal communities on anodes of PMFC with rice field soil rather than non-endogenous soil substratum was studied in order to identify those microorganisms that are likely involved in current generation. Therefore, total RNA was extracted from duplicate anodes and bulk soil samples from both series of PMFCs, as well as the controls at the end of the operation time (as described previously by Lueders *et al.*, 2003). After reverse transcription, terminal restriction fragment length polymorphism (TRFLP) fingerprints of the bacterial and archaeal 16S rRNA were generated from all anode and bulk soil samples (see Appendix S1 for detailed materials and methods). For the identification of the bacterial populations, three clone libraries were generated for series A, i.e. one from a duplicate PMFC, one from the OC control and one from the unplanted control (see Appendix S1 for detailed materials and methods). Similarly, one clone library was constructed from the archaeal 16S rRNA PCR product from the same PMFC used for the bacterial clone library. Sequence data of retrieved bacterial and archaeal 16S rRNA gene fragments were used for the phylogenetic tree construction and for the *in silico* terminal restriction fragments (TRFs) assignments. Sequence data were deposited in GenBank under accession numbers JF325892–JF326184.

Active bacterial populations on anodes

TRFLP analysis of bacteria indicated stark differences between the active bacterial community of PMFCs compared with OC and NP controls and bulk soil (Fig. 1A and B). Thus, specific microorganisms were selected on the anode when current was produced (PMFC versus OC) as well as when rhizodeposits were present as substrate (PMFC versus NP). The main difference detected between PMFCs and OC was the increase of the relative abundance of a 159 bp TRF (Fig. 1A), which increased from 9% in the OC control up to 24% in the PMFC, and was not detectable in the bulk soil sample. Second most

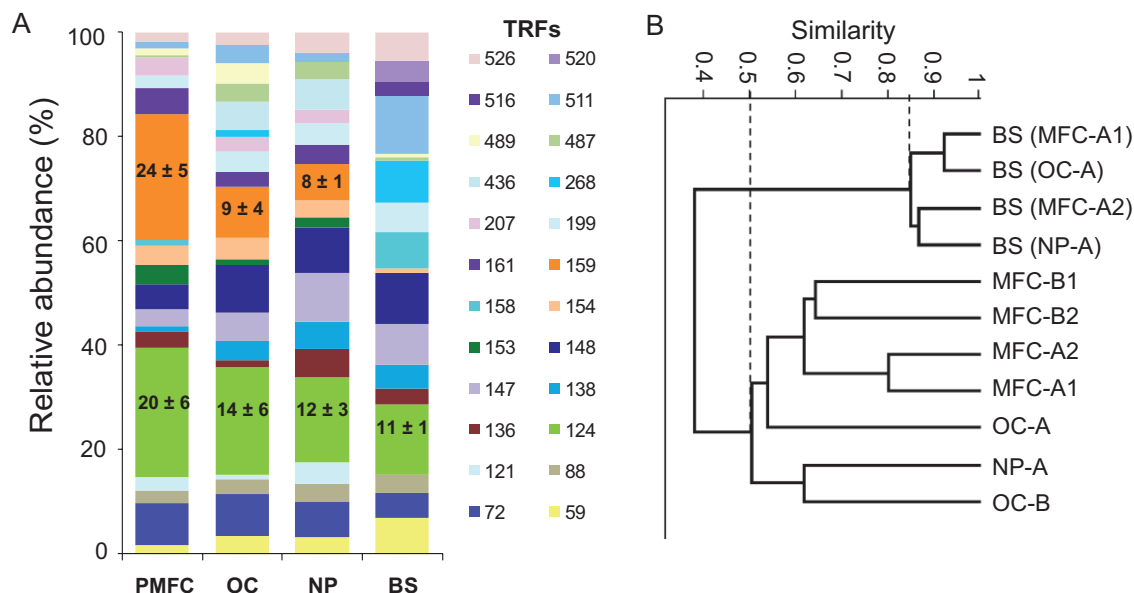


Fig. 1. TRFLP analysis of bacterial 16S rRNA on anodes from rice microbial fuel cell (PMFC), unplanted control (NP), open circuit control (OC) and bulk soil samples (BS).

A. TRF size and relative abundance (%) for each sample. On the right of the graph the sizes of the TRFs are shown in base pairs. Relative abundance of TRFs 159/161 bp (*Geobacter* spp.) and 124 bp (*Anaeromyxobacter* spp.) was added to the figure with the corresponding standard deviation.

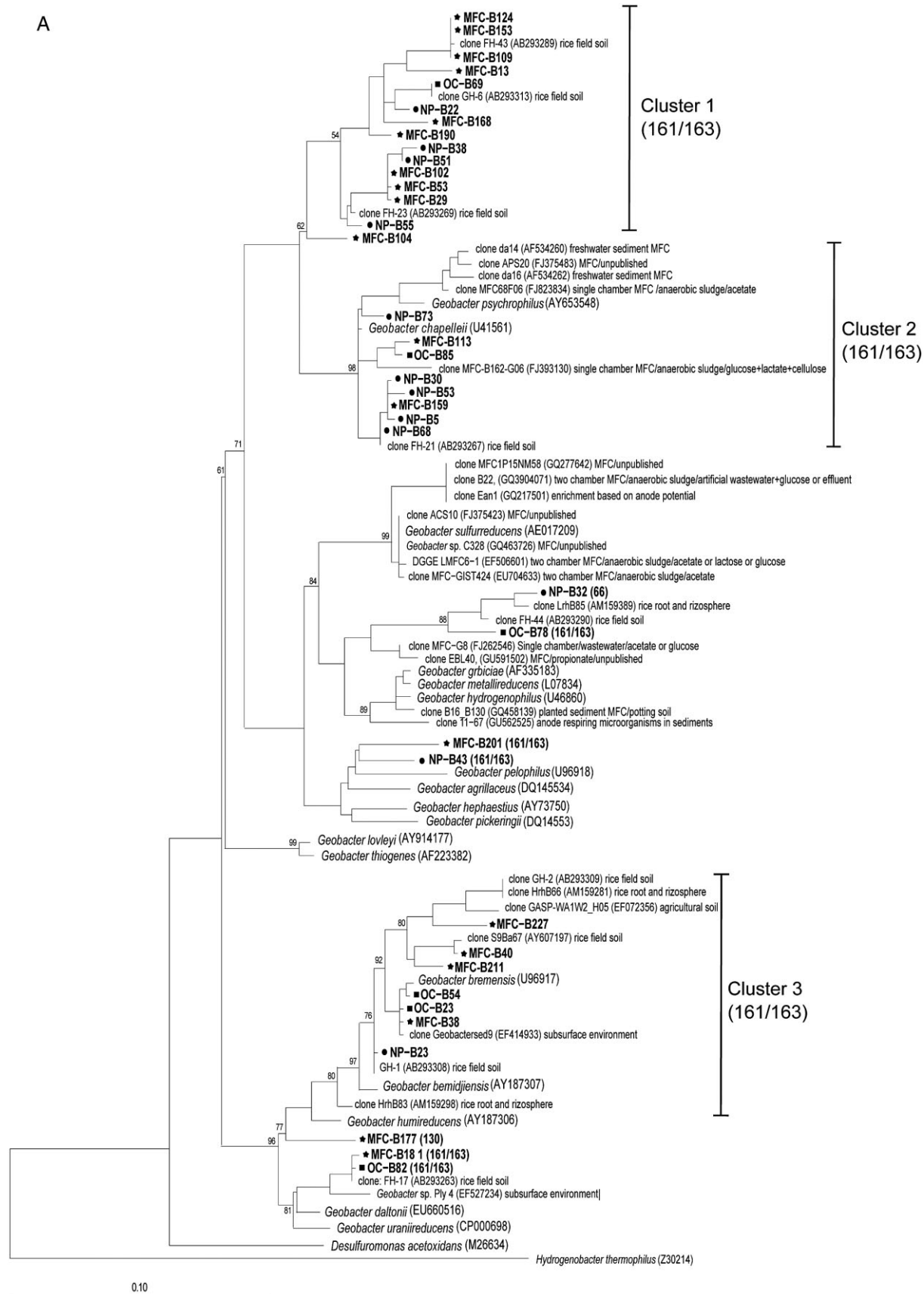
B. Cluster analysis performed for the TRFLP data. PMFC: $n = 4$, NP: $n = 2$, OC: $n = 3$, BS: $n = 4$. TRFs with less than 2% relative abundance (in total 42 TRFs with total average relative abundance of 22%) were not included in the graphic representation and were not associated to *Anaerolinea*, *Geobacter* spp. or *Anaeromyxobacter* spp. Cluster analysis was performed with PAST software using the algorithm UPGMA and the Bray–Curtis similarity index. Dotted lines indicate the intragroup similarities.

abundant was a 124 bp TRF on PMFC anodes; however, this TRF was also abundant in all samples analysed. The relative abundance of TRFs present on anodes from OC samples decreased in the PMFC (TRFs of 489 bp, 436 bp, 148 bp, 138 bp), indicating a stimulation of few populations only on current producing anodes (Fig. 1A).

Cloning and sequence analysis of 16S rRNA revealed differences in the composition of the active bacterial communities in each of the anode samples analysed. On PMFC anode samples, clones related to *Delta-proteobacteria* were more abundant compared with control samples, representing almost 50% of all clone sequences (Table S1). Within this group, most of the clones were related to the family *Geobacteraceae* and the order *Myxococcales* (Fig. 2A and B, Table S1). Clone sequences related to *Geobacter* (92–99% sequence identity, Table S2) were highly abundant in PMFCs and NP control compared with OC controls, indicating the stimulation of *Geobacter*-related populations in current producing systems (Table S1, Fig. 2A). *Geobacter sulfurreducens* and *G. metallireducens* are known anode-reducing bacteria (Bond *et al.*, 2002; Bond and Lovley, 2003), and *Geobacter* spp. were found abundant on anodes of two-chamber MFCs, marine sediment MFCs and PMFCs (Holmes *et al.*, 2004b; Jung and Regan, 2007; Timmers *et al.*, 2012). *Geobacter*-related clones fell

into three main clusters (clusters 1, 2 and 3 in Fig. 2A) with an *in silico* TRF of 161/163 bp (Table S1, Fig. 2A). The high clone abundance of cluster 1-related sequences in PMFC (11%) compared with the unplanted control (6%) suggests a stimulation of this particular *Geobacter* population by the presence of the plant (Fig. 2A).

Myxococcales-related sequences were more abundant in PMFCs and OC controls than in the NP control and grouped within two larger clusters (clusters 4 and 5, Fig. 2B, Table S2). Clones with an *in silico* TRF of 129 bp fell into the larger radiation of *Anaeromyxobacter* spp., and most clone sequences were related to *Anaeromyxobacter dehalogenans* strain 2CP-3 (cluster 4, Fig. 2B, Table S2). *Anaeromyxobacter*-related sequences grouped in three main clusters (clusters 4A, 4B and 4C in Fig. 2B). Cluster 4B clones were more abundant (25% in PMFC versus 5% in OC-A) on the anode from PMFCs compared with the OC control (Fig. 2B), suggesting that within the genus *Anaeromyxobacter* these populations might be able to transfer electrons to anodes. Moreover, cluster 4B clone sequences were not detected in the unplanted control, indicating a selection of these *Anaeromyxobacter* spp. on anodes of PMFCs (Fig. 2B). *Anaeromyxobacter* spp. had so far not been found on anodes of MFCs, and nothing is known about their capacity to transfer electrons to an anode, either directly



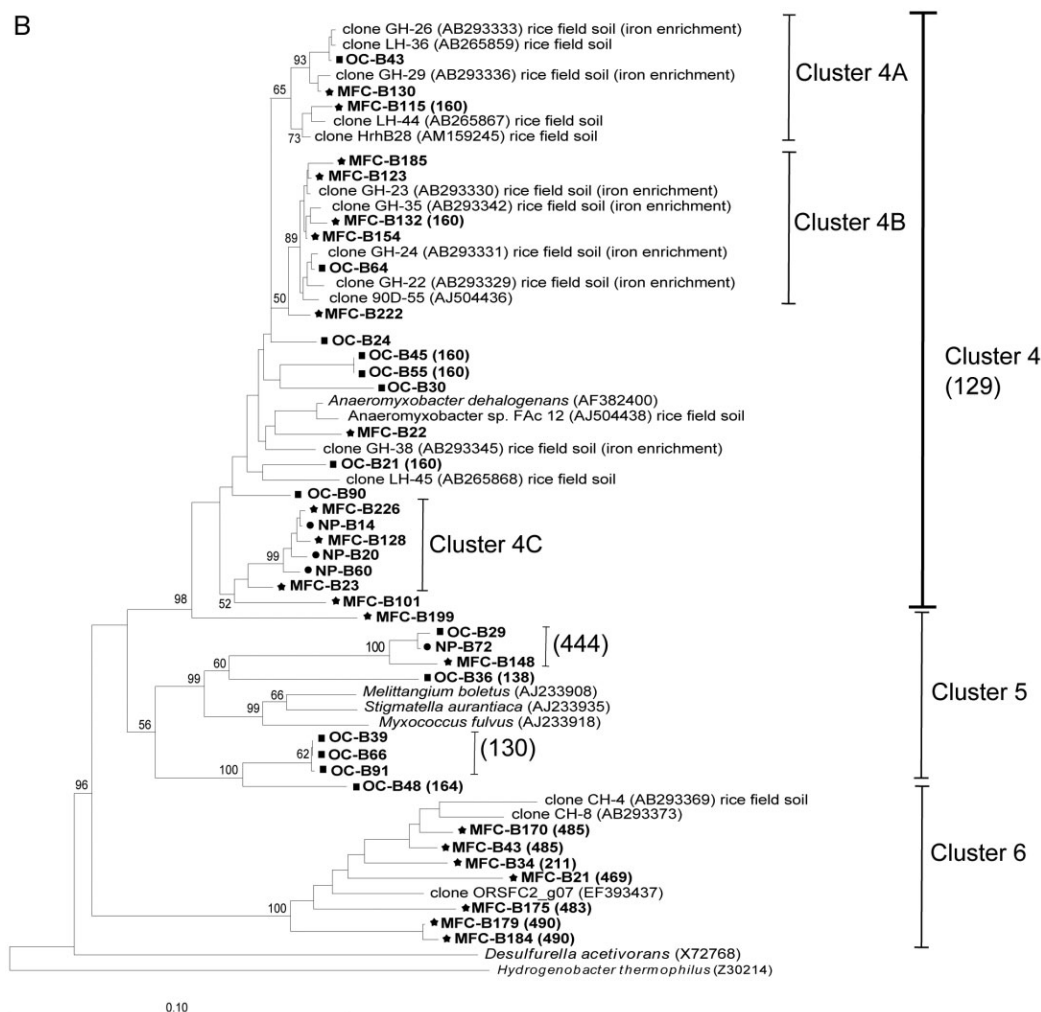


Fig. 2. Phylogenetic trees showing the relationships of 16S rRNA clone sequences related to *Geobacter* spp. (A) *Anaeromyxobacter* spp. and uncultured *Deltaproteobacteria* (B), and *Chloroflexi* (C). Clones obtained in this study from planted sediment microbial fuel cell (MFC: star), unplanted control (NP: circle) and open circuit control (OC: square) were included in the phylogenetic trees. The TRF sizes are as indicated in brackets in base pairs. Bootstrap values were obtained from 1000 replications. The scale bar represents 10% sequence divergence. GenBank accession numbers of reference sequences as indicated. Subphylum I and II in the *Chloroflexi* phylogenetic tree is according to Yamada and Sekiguchi (2009).

or by using shuttles (Rabaey *et al.*, 2005). However, *Anaeromyxobacter* populations abundantly occur on rice roots, and the iron (III)-reducing *Anaeromyxobacter* strain FAc12 was isolated from rice field soil (Treude *et al.*, 2003). It has been shown by RNA-SIP analysis that *Anaeromyxobacter* populations actively incorporate ^{13}C -acetate in the presence of ferrihydrite and goethite (Hori *et al.*, 2010), which are closely related (98–99% sequence identity) to those *Anaeromyxobacter* clones found on the anode (Fig. 2B). Still, *Anaeromyxobacter* isolates have to be tested for an unequivocal proof of anode-reducing capability, as the capability of reducing iron oxides does not necessarily imply the ability to transfer electrons to anodes (Richter *et al.*, 2007).

A group of clones from PMFCs formed a separate cluster within the *Deltaproteobacteria* (cluster 6; Fig. 2B) and were closely related to environmental clone sequences from rice field soil. These clone sequences were not detected among unplanted and OC controls (Table S1, Fig. 2B), indicating a strong influence of current and rice root exudates on this population.

Another group of bacteria abundant on anodes of PMFC belonged to phylum *Chloroflexi* and were twice as abundant as in the OC control. This group was also abundant in the unplanted control and grouped in two clusters (clusters 7 and 8, Table S1, Fig. 2C) with rather diverse *in silico* TRFs, which hindered peak assignment in TRFLP profiles. The majority of *Chloroflexi*-related sequences

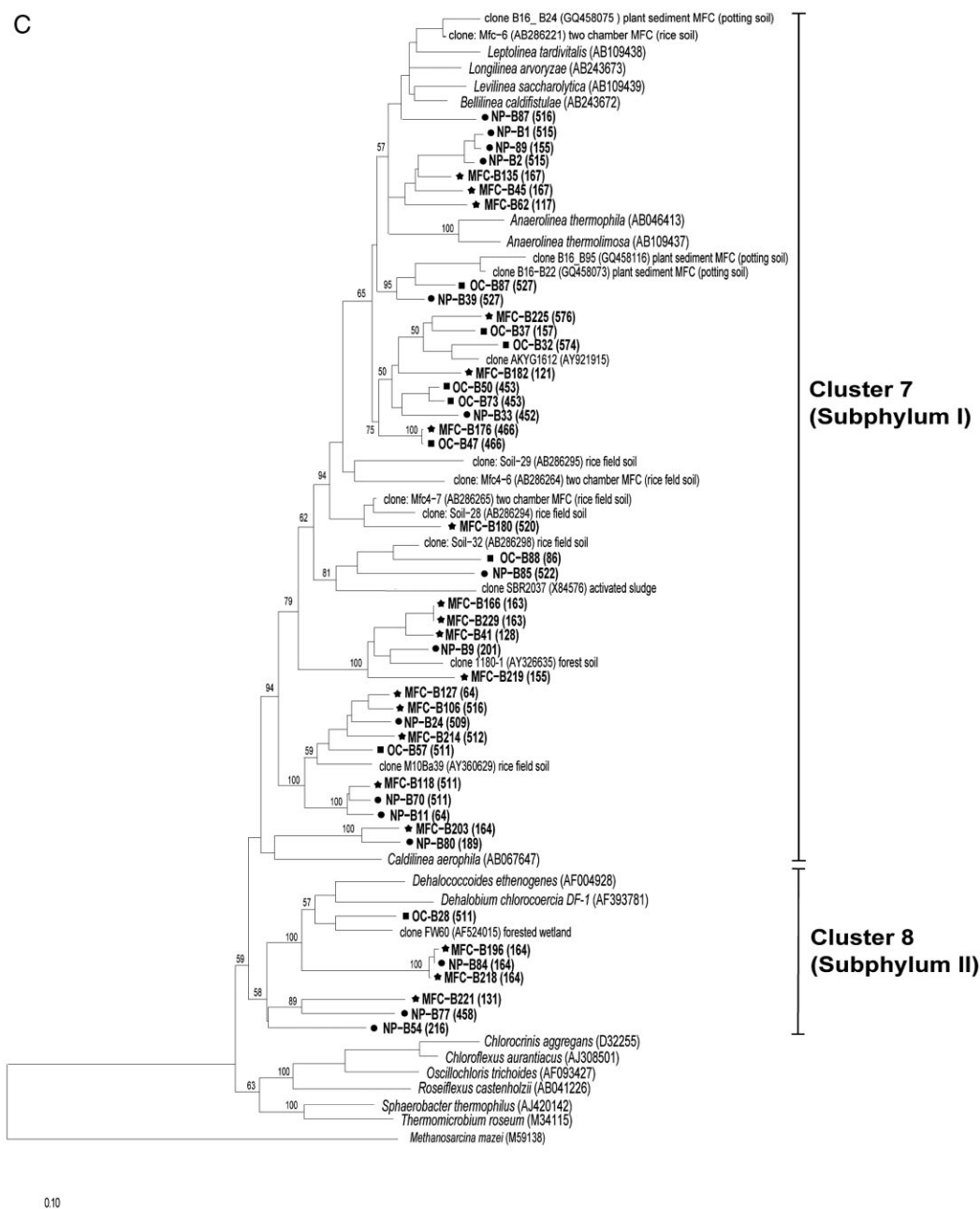


Fig. 2. cont.

from PMFC and unplanted MFC anode samples fell into the *Anaerolineae* lineage (cluster 7), indicating a stimulation of these populations on current producing anodes. *Anaerolineae* have scarcely been found in MFCs; they appear to be important in MFCs inoculated with soil, in particular rice field soil (Ishii *et al.*, 2008b; this work). With potting soil as substratum in PMFCs, 6% of all clones were *Anaerolineae* (De Schampelaire *et al.*, 2010); however, their proportion in rice field soil PMFCs was much higher (17% of all clones), and also Ishii and

colleagues (2008b) observed an enrichment of up to 22% of *Chloroflexi* sequences on the anode of a two-chamber MFC inoculated with rice field soil. The *Anaerolineae* lineage still contains surprisingly diverse, yet to be cultured, environmental clades (Yamada and Sekiguchi, 2009). The cultured representatives of these lineages are filamentous, slow-growing, and aerobic and anaerobic heterotrophs decomposing carbohydrates and amino acids (Yamada and Sekiguchi, 2009). The uncultured *Anaerolineae* detected appear to be involved in electron

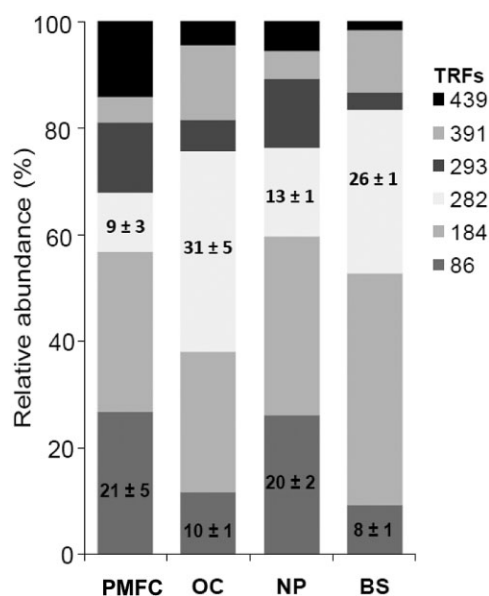


Fig. 3. Archaeal community analysis. TRFLP analysis of archaeal 16S rRNA on anodes from planted sediment microbial fuel cells (PMFC), unplanted controls (NP), open circuit controls (OC) and bulk soil samples (BS). Shown are TRF relative abundance averages (%) for each sample analysed (PMFC: $n = 4$, NP: $n = 2$, OC: $n = 3$, BS: $n = 4$). TRFs with less than 5% relative abundance were not included in the graphic representation. On the left of each graph the sizes of the TRFs are shown in base pairs. Most detectable TRFs have already been assigned to defined archaeal lineages for rice field soil (Lueders *et al.*, 2004). Based on the clone library, well-known methanogenic archaea were identified, e.g. *Methanomicrobiales* (86 bp TRF, 391 bp TRF), *Methanosarcinales* (184 bp TRF), *Methanosaetaceae* (282 bp TRF), and *Methanocellales* (391 bp TRF). Relative abundance of TRFs 86 bp (*Methanobacteriales*) and 282 bp (*Methanosaeta* spp.) was added to the figure with the corresponding standard deviation.

transfer to anodes; however, it is presently unclear whether they are directly involved or whether they produce metabolic intermediates from root exudates or soil organic matter, utilized subsequently by other directly anode-coupling microorganisms.

Effect of closed circuit PMFC on the methanogenic community

Plant-based sediment microbial fuel cells have been suggested as a methane emission mitigation option as introducing an anode in the rhizosphere of waterlogged plants possibly by favouring anode respiring metabolism which competes with the commonly present methanogenic metabolism (De Schamphelaire *et al.*, 2010; Arends *et al.*, 2014). Here, we detected differences in the archaeal community composition on PMFC anodes compared with OC controls and bulk soil samples, as shown by TRFLP as well as cloning, and sequencing of 16S rRNA transcripts (Fig. 3 and Table 1, Fig. S2). On anodes from the PMFC, the abundance of active

acetoclastic *Methanosaeta* spp. (TRF 282 bp) decreased, whereas active hydrogenotrophic *Methanomicrobiales* (TRF 82 bp) increased, suggesting a shift from acetotrophic to hydrogenotrophic methanogenesis. Possibly, anode-reducing *Geobacter* populations compete with methanogens for the common substrate acetate; however, acetate concentrations in open and closed circuit systems have to be determined to prove this hypothesis. Similar shifts in archaeal community composition were observed in rice PMFCs with potting soil (De Schamphelaire *et al.*, 2010) and graphite granules (Arends *et al.*, 2014). Moreover, Ishii and colleagues (2008a) detected less methanogens on the anode and suppressed methanogenesis in a two-chambered MFC inoculated with 1% rice field soil compared with OC control anodes. The shift in methanogen community structure indicates that the redox conditions were not adverse for the activity of methanogens in general. Methanogens have been shown to accept electrons from a cathode to produce methane, indicating an ability of methanogens to directly interact with electrodes in fuel cell systems (Cheng *et al.*, 2009). Regarding the effect of MFCs on methane production, more research is needed to demonstrate the effect of introducing an anode to a paddy soil on methane emissions. Arends and colleagues (2014) showed that methanogenesis was delayed when an anode was introduced into a waterlogged rhizosphere; however, a long-term effect could not be maintained due to the excess of organic matter. The presence of an anode in a rice paddy soil might also reduce or delay methane production and might be an approach to mitigate methane emission from rice paddies.

Conclusions

Differential analysis of PMFCs, unplanted and OC controls, allowed to delineate populations selectively enriched on anodes of PMFCs. A predominance of *Geobacter* populations and also a group of unclassified *Deltaproteobacteria* as well as *Anaeromyxobacter*

Table 1. Phylogenetic affiliation and relative proportion of archaeal 16S rRNA clone sequences from the PMFC anode sample (PMFC-A2).

Phylogenetic group	Clones (%) ($n = 61$)
<i>Methanosarcinales</i>	34.4
<i>Methanosaetaceae</i>	26.2
<i>Methanocellales</i> (Rice cluster I)	16.4
<i>Methanomicrobiales</i>	14.8
Rice cluster IV	3.3
Uncultured <i>crenarchaeote</i>	1.6
Uncultured <i>euryarchaeote</i>	1.6
Uncultured <i>Thermoplasmatales</i>	1.6

populations and *Anaerolineae* was detected on anodes of rice field soil PMFCs. Our data suggest that the release of root exudates into the PMFC system selectively enriched for distinct populations of *Geobacter*, *Anaeromyxobacter* and unclassified *Deltaproteobacteria*. However, it is still not clear whether root exudates might be directly converted into current or first degraded into intermediate compounds such as acetate by fermenting bacteria, which would then serve as fuel for current production. Competition for acetate might also explain a decrease in relative abundance of acetate-utilizing *Methanosarcinales* in current producing PMFCs. When current was produced, clear changes in the bacterial and archaeal community compositions were observed, and factors such as plant presence and inoculum seem important in determining the composition of active microorganisms on anodes. Further experiments, e.g. using a stable isotope probing approach, would be required to get more insight into the interaction between root exudates and anode-reducing bacteria.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Current density (mA m⁻² TAS) profiles of planted (filled square: PMFC-A, filled triangles: PMFC-B) and unplanted control (filled circle: NP-A) during operation time.

Fig. S2. Phylogenetic tree showing the relationships of 16S rRNA clone sequences related to Archaea. Clones obtained in this study from planted sediment microbial fuel cell (MFC) were included in the phylogenetic trees. The *in silico* TRF sizes are as indicated in brackets in base pairs. Bootstrap values were obtained from 1000 replications. The scale bar represents 2% sequence divergence. GenBank accession numbers of reference sequences as indicated.

Table S1. Clone sequence analysis and TRF assignment. The table shows phylogenetic affiliations, *in silico* and *in vitro* TRF length, and relative abundance of 16S rRNA clones retrieved from plant microbial fuel cells (PMFC), open circuit control (OC) and unplanted control (NP).

Table S2. Sequence identity in percentage obtained from the similarity matrix of clone clusters 1–8 depicted in the phylogenetic trees (Fig. 2).

Appendix S1. Materials and methods.